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Susceptibility of Geranium Cultivars to *Ralstonia solanacearum*

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Abstract. **Sixty-one cultivars of geraniums, including zonal, regal, ivy, and scented, were tested for susceptibility to three strains of *Ralstonia solanacearum*: a race 1, biovar 1 (R1B1) strain P597 isolated from tomato in Florida, a R1B1 strain P673 obtained from pothos originated from Costa Rica, and a race 3, biovar 2 (R3B2) strain UW551 isolated from geranium imported from Kenya. These three strains represent populations of *R. solanacearum* found in the United States or imported with infected plant propagative material. A genomic comparison of the geranium cultivars was also done using amplified fragment length polymorphisms. Both R1B1 strains were more virulent than the R3B2 strain, producing wilt symptoms on most cultivars of zonal, regal, and ivy types. Variation in susceptibility of geranium cultivars to the two R1B1 strains was observed. The R3B2 strain UW551 had a much more restricted host range and was not able to infect most regal geranium cultivars when applied as a soil drench. Many of the scented cultivars were found to be resistant to all three strains of *R. solanacearum* when tested using the drench inoculation method. However, most scented cultivars were found to be susceptible when plants were wound-inoculated. The greatest variation in type of resistance was observed between the scented geranium cultivars and specific strains of *R. solanacearum*.**

Geraniums (*Pelargonium* spp.) have been cultivated for more than 350 years and over 200 species have been grown during this time period (Miller, 2002). Hybridization and selections have transformed this potted flowering crop into one of the most popular bedding plants worldwide. The wide variety of cultivars varies extensively in growth habit, flower structure, floral color, and foliage and are generally grouped into four types: zonal (*Pelargonium ×hortorum*), ivy (*Pelargonium peltatum*), regal (*Pelargonium ×domesticum*), and scented (*Pelargonium* spp.).

Many commercial propagation facilities produce cultivars of zonal, regal, ivy, and scented geraniums within the same location. Geranium production is labor-intensive, especially during early stages of propagation. Therefore, geranium propagation is outsourced to countries with low labor costs. Endemic populations of *Ralstonia solanacearum* (Yabuuchi et al., 1996), the causal agent of bacterial wilt disease, exist in most of these countries and pose a significant risk.

Currently there are three classification systems commonly used to distinguish populations of *R. solanacearum*. Populations are broken into five races based on host range (Buddenhagen et al., 1962; He et al., 1983; Pegg and Moffet, 1971); six biovars based on biochemical properties (Hayward, 1964, 1991); and into 23 sequevars based on endoglucanase gene sequence (Fegan and Prior, 2005). All established populations of *R. solanacearum* found in the southern United States so far have been classified as race 1 and can be separated into biovars 1 and 3 and sequevars 4, 5, 7, and 13 (Ji et al., 2007). *Ralstonia solanacearum* race 3, biovar 2, sequevar 1 strains cause losses in potato and tomato production in tropical regions of the world and have more recently been associated with geranium production. Isolates found on geranium have been shown to be pathogenic on potato (Williamson et al., 2002) and because of their ability to infect potato and survive in temperate climates, race 3 biovar 2 strains have been placed into the National Select Agent List. In Europe, race 3 biovar 2 strains have become established in potato production (Janse et al., 2004). Race 3 biovar 2 strains have been introduced multiple times into the United States through infected geranium cuttings (Kim et al., 2003; Norman et al., 2009; Williamson et al., 2002). Eradication of race 3 strain in the United States has always been successful. There has also never been a documented case in which infected geraniums have been shown to be the source of inoculum for any outbreak in potato production. Because of the potential threat of the introduction of bacterial wilt, numerous polymerase chain reaction assays have been developed for the detection of *R. solanacearum* in soil, water, and plant samples (Caruso et al., 2003; Jung et al., 2007; Kutin et al., 2009; Marco-Noales et al., 2008; Ozakman and Schaad, 2003; Poussier et al., 2002; Schönfeld et al., 2003).

The objective of this research was to determine which types of geraniums are most susceptible to bacterial wilt, to identify the range of cultivar resistance to the disease, and to evaluate which cultivar types could harbor bacterial wilt pathogens as asymptomatic carriers.

Materials and Methods

Sixty-one cultivars of geraniums were used in this study with representatives from all four geranium types (Table 1). Because of the extensive hybridization of geraniums, a genomic comparison of the 61 cultivars was done using amplified fragment length polymorphism (AFLP) analysis. The procedure was performed with the *Eco*RI and *Mse*I restriction enzymes with an IRDye System (Janssen et al., 1996). Selective amplifications were performed using M-CAT/E-ACC primers (Chen et al., 2004). Fragments were resolved on acrylamide gels run for 4 h at 1500 V on a Global LI-COR IR^2 System (Lincoln, NE). Images were recorded using the SAGA software (LI-COR). Tiff files were analyzed with the BioNumerics program (Version 2.1; Applied Maths, Kortrijk, Belgium) using the unweighted pair group method with arithmetic mean.

Three strains of *R. solanacearum* were used for cultivar susceptibility screening, including two race 1, biovar 1 (R1B1) strains, P597 and P673, and a race 3, biovar 2 (R3B2) strain UW551. P597 was isolated in Florida from tomato, whereas P673 was obtained from unrooted cuttings of pothos imported from Costa Rica (Norman et al., 2009). UW551 was isolated from geranium cuttings

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Table 1. Response of 61 geranium cultivars (*Pelargonium* sp.) to three *R. solanacearum* strains causing bacterial wilt by drench inoculation or by wound inoculation.

	Drench inoculation[z]			Wound inoculation		
	Ralstonia strain			*Ralstonia* strain		
Geranium type	R1B1 P597	R1B1 P673	R3B2 UW551	R1B1 P597	R1B1 P673	R3B2 UW551
Zonal (*Pelargonium ×hortorum*)						
Cultivar						
Americana Dark Red	S	S	S	—	—	—
Americana Light Salmon	S	S	S	—	—	—
Americana Pink III	S	S	R	—	—	S
Americana Red	S	S	S	—	—	—
Americana White	S	S	S	—	—	—
Americana White Splash II	S	S	S	—	—	—
Brocade Vancouver Centennial	S	S	S	—	—	—
Candy Bright Red (Fireball)	S	S	S	—	—	—
Designer Bright Lilac Imp	S	S	S	—	—	—
Designer Hot Pink	S	S	S	—	—	—
Designer Bright Red Imp	S	S	S	—	—	—
Designer Dark Red	S	S	S	—	—	—
Designer Salmon	S	S	S	—	—	—
Designer White	S	S	S	—	—	—
Eclipse Red III	S	S	S	—	—	—
Eclipse Rose	S	S	R	—	—	S
Fantasia Cardinal Red Imp	S	S	S	—	—	—
Fantasia Flamingo Rose Imp	S	S	S	—	—	—
Fantasia Pink Shell	S	S	S	—	—	—
Fantasia Salmon	S	S	S	—	—	—
Galleria Bright Violet	S	S	S	—	—	—
Galleria Pink Punch	S	S	S	—	—	—
Patriot Bright Red	S	S	S	—	—	—
Patriot Red	S	S	S	—	—	—
Patriot Bright Violet	S	S	S	—	—	—
Patriot White	S	S	S	—	—	—
Pink X2	S	S	S	—	—	—
Rocky Mountain Dark Red	S	S	S	—	—	—
Showcase Red	S	S	S	—	—	—
Showcase Scarlet	S	S	S	—	—	—
Showcase White	S	S	S	—	—	—
Stardom Deep Lavender	S	S	S	—	—	—
Ivy (*Pelargonium peltatum*)						
Cultivar						
Beach 99	S	S	S	—	—	—
Colorcade Burgandy Ice	S	S	S	—	—	—
Colorcade Purple	S	S	S	—	—	—
Colorcade Red Imp	S	S	S	—	—	—
Dark Red Blizzard	S	S	S	—	—	—
Freestyle Lavender II	S	S	S	—	—	—
Freestyle White	S	S	R	—	—	S
Global Merlot	S	S	S	—	—	—
Nicole	S	S	S	—	—	—
Sybil Holmes	S	S	S	—	—	—
Regal (*Pelargonium ×domesticum*)						
Cultivar						
Elegance Baroness	S	S	R	—	—	S
Elegance Camelot	S	R	R	—	S	S
Elegance Pink Chiffon	S	S	R	—	—	S
Maiden Red	S	S	S	—	—	—
Scented (*Pelargonium* spp.)						
Cultivar						
Apricot (*P. scabrum*)	R	R	R	S	R	R
Balsam (*P.* sp.)	S	R	S	—	S	—
Chocolate Mint (*P. quercifolium*)	R	R	R	S	C	S
Citronella (*P. citrosum*)	R	R	R	C	C	R
Ginger (*P.* sp.)	S	S	R	—	—	S
Lemon (*P. crispum*)	S	R	S	—	S	—
Lime (*P. nervosum*)	S	R	R	—	S	R
Mint-Scented Rose (*P. graveolens*)	R	R	R	S	S	R
Old Fashiond Rose (*P. graveolens*)	R	R	R	S	C	R
Peppermint Rose (*P. graveolens*)	R	R	R	C	C	R
Pine (*P. denticulatum*)	S	R	S	—	S	—
Rober's Lemon Rose (*P. graveolens*)	R	R	R	S	—	R
Round Leaf Orange (*P.* sp.)	R	S	R	S	—	C
Snowflake (*P. capitatum*)	R	R	R	S	R	R
Strawberry (*P. ×scarboroviae*)	S	S	S	—	—	—
Control						
Tomato (*Solanum lycopersicum*)	S	S	S	S	S	S

[z]S = susceptible, plants are systemically infected and wilted; R = resistant, no wilt symptoms or systemic infections observed; C = carriers (asymptomatic), plants showed no wilt symptoms but tested positive for systemic infection.
— = not tested.

imported from Kenya and was provided by C. Allen, University of Wisconsin. Both UW551 and P673 were found in previous research to possess cold shock genes (Duan et al., 2005) as well as genes that enable the pathogen to infect and cause plant wilt at 18 °C (Norman et al., 2007). Both of these strains represent populations of *R. solanacearum* that entered the United States in infected plant propagative material (Norman et al., 2009).

Uniform stock pots of all cultivars of geraniums were established in the Plant Pathology Research Greenhouses at the Mid-Florida Research and Education Center, Apopka, FL. Zonal, ivy, and regal geraniums were obtained as plugs and were potted into 0.5-L pots filled with Vergro Container Mix A, which includes 60% Canadian peat, 20% vermiculite, and 20% perlite (Verlite Co., Tampa, FL). Scented geraniums were received as unrooted cuttings. The cuttings were stuck directly into 0.5-L pots containing Vergro Container Mix A and rooted under intermittent mist until rooting was completed before they were moved into the greenhouse or growth chambers. All plants subject to R1B1 testing were placed in a greenhouse with temperatures maintained between 18 and 32 °C, maximum lighting at 266 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and a natural photoperiod. Osmocote-Plus ($15N–9P_2O_5–12K_2O$; Sierra Chemical Co., Inc., Milpitas, CA) was applied in a single initial planting application at 1.5 g/pot. Bacterial inoculations were conducted during a 9-month growing season extending from Mar. to Nov. 2007. Ten uniform plants of each cultivar were selected at random from established blocks for inoculation. Flowers were removed before testing, but no blooms were removed during experiments.

Because the R3B2 strain UW551 is a Select Agent pathogen listed by USDA/APHIS, susceptibility of geraniums to this bacterium was tested in secured environmental chambers set on a 12-h day/night cycle (19 °C night, 24 °C day, 310 $\mu mol \cdot m^{-2} \cdot s^{-1}$).

Two inoculation methods were used. The first method consisted of drench inoculation (50 mL) poured onto the soil surface. Inoculum was prepared by suspending 24-h-old bacterial cultures in saline (8.5 $g \cdot L^{-1}$ NaCl) and spectrophotometrically adjusting (A_{600}) the concentration to 1×10^7 cfu/mL, resulting in a final concentration in the soil of 6×10^6 cfu/g.

Wilt dates were recorded over a 60-d period. At the end of the 60 d, a cross-section of stem 1 cm in length was removed ≈0.5 cm above the soil line from the first three replicate geranium plants of each cultivar. If no *R. solanacearum* was reisolated from the first three plants, other samples were taken from any replicate plants with wilt symptoms. Stem sections were dipped in a 10% bleach solution (0.3% NaOCl), blotted, and ground in 300 µL of sterile distilled water. Tissue suspensions were dilution plated onto triphenyltetrazolium chloride medium (Kelman, 1954) and incubated at 28 °C for 48 h Plates were examined for colonies having the typical mucoid red swirling egg-shaped pigmentation pattern of *R. solanacearum* previously described by Kelman (1954). Each cultivar was assigned one of the following three ratings for each of the three *R. solanacearum* strains: susceptible (S), plants became systemically infected and wilted; resistant (R), no wilt symptoms or systemic infections observed; and carrier (C), asymptomatic plants without wilt symptoms but tested positive for systemic infection.

Large numbers of *R. solanacearum* cells can be found in the root zone of infected zonal geraniums (Swanson et al., 2005). Bacterial cells are associated with infected roots and substantial amounts are released into media when infected plants decompose. Bacterial populations in the root system can serve as a secondary source of inoculum within a nursery. To assess if there was a difference in soil populations between geranium types, three soil samples were taken from each cultivar/isolate combination at termination of testing. Soil sample was taken by vertically inserting a cork borer (#10, 15 mm diam.) into planting medium from the surface to the bottom of the pot. Media recovered was mixed and a 1-g subsample was taken from each of the three soil samples. Using these subsamples, a dilution series of the media was done in sterile distilled water and replica-plated onto modified semiselective medium South Africa agar (Elphinstone et al., 1996). Typical *R. solanacearum* colonies were counted after incubation at 28 °C for 48 h. To confirm that colonies were counted properly, selected colonies from typical *R. solanacearum* colonies were restreaked for purity, incubated for 24 h, suspended in saline (8.5 $g \cdot L^{-1}$ NaCl), and injected into parenchymatous tissue of tobacco (*Nicotiana tabacum*, 'Hicks') for hypersensitive response (HR) following established protocols (Lozano and Sequeira, 1970). A positive HR reaction is characterized by death of infiltrated tobacco cells within 24 h confirming host recognition of pathogen.

Cultivars that did not become infected by using the first inoculation method were tested further using a wound inoculation method. Geranium plants (n = 10) were grown to ≈17 cm in height. The growing tip of each plant was excised. The cut surface of each stem was inoculated by pipetting 20 µL of a cell suspension containing between 1000 and 5000 cfu of *R. solanacearum*. Inoculum concentration was confirmed by dilution plating.

After 60 d, two isolations were made from stem sections of all plants. The first sample was taken 1 cm down from the inoculation point and the second sample ≈16 cm down at soil level. Each sample consisted of a stem cross-section ≈0.5 g in weight. Previously outlined isolation methods were used for sample processing. Tests of R1B1 strains were again done under greenhouse conditions, whereas those of the R3B2 strain in environmental chambers set on a 12-h day/night cycle (24 °C night, 28 °C day, 310 $\mu mol \cdot m^{-2} \cdot s^{-1}$). Temperatures in growth chambers for tests using the wound inoculation method were increased to increase infection of R3B2 strain (Swanson et al., 2005) and to more closely reflect temperatures in the greenhouse study. Geranium susceptibility tests were repeated under the same environmental conditions. For all greenhouse and growth chamber experiments, a saline control was used as a noninoculated geranium control. Tomato plants were inoculated with each of the three strains of *R. solanacearum* to serve as the positive controls in the experiments.

Results

Zonal and ivy cultivars were shown by AFLP analysis to be closely related with most cultivars having a 90% or greater similarity coefficient (Fig. 1). A far greater genetic diversity was seen in cultivars of regal and scented geranium types. Low similarity coefficients are expected because many of theses cultivars represent individual species that are probably not capable of interspecific crosses.

All 32 zonal and 10 ivy cultivars were found to be susceptible to both R1B1 strains when the drench inoculation method was applied. Symptom expression was rapid with most plants exhibiting wilt symptoms within 2 weeks of inoculation with either P597 or P637. The R3B2 strain UW551 was less aggressive with some zonal and ivy cultivars having as low as a 10% infection rate. Two zonal cultivars, Americana Pink III and Eclipse Rose, and one ivy cultivar, Freestyle White, did not become systemically infected with the R3B2 strain (Table 1). On further testing using the wound inoculation method, however, all of the three cultivars were shown to be susceptible to UW551. There was no significant difference ($P = 0.05$) in soil populations of any of the three *Ralstonia* strains within pots of zonal and ivy geraniums (Table 2). There was some variability in bacterial populations between individual cultivars that was found to be related to the disease progress observed with each plant.

By soil drench inoculation, all four tested regal geranium cultivars (*Pelargonium ×domesticum*) were susceptible to the R1B1 strain P597, and three of the four cultivars were also susceptible to the other R1B1 strain P673 (Table 1). The regal cultivars were very resistant to the R3B2 strain UW551, however, because three of the four cultivars did not show any wilt symptoms nor contained UW551 in their stems. When the three regal cultivars were tested further by wound inoculation with UW551, all were found to be susceptible. Symptoms were, however, milder than those seen on zonal or ivy cultivars with only mild chlorosis and leaf curl. Strain UW551 was not reisolated from the soil (Table 2) and roots of drench-inoculated regal cultivars (data not shown).

The greatest variability in cultivar susceptibly to *R. solanacearum* was observed among scented geranium varieties. Many scented geranium cultivars showed a high

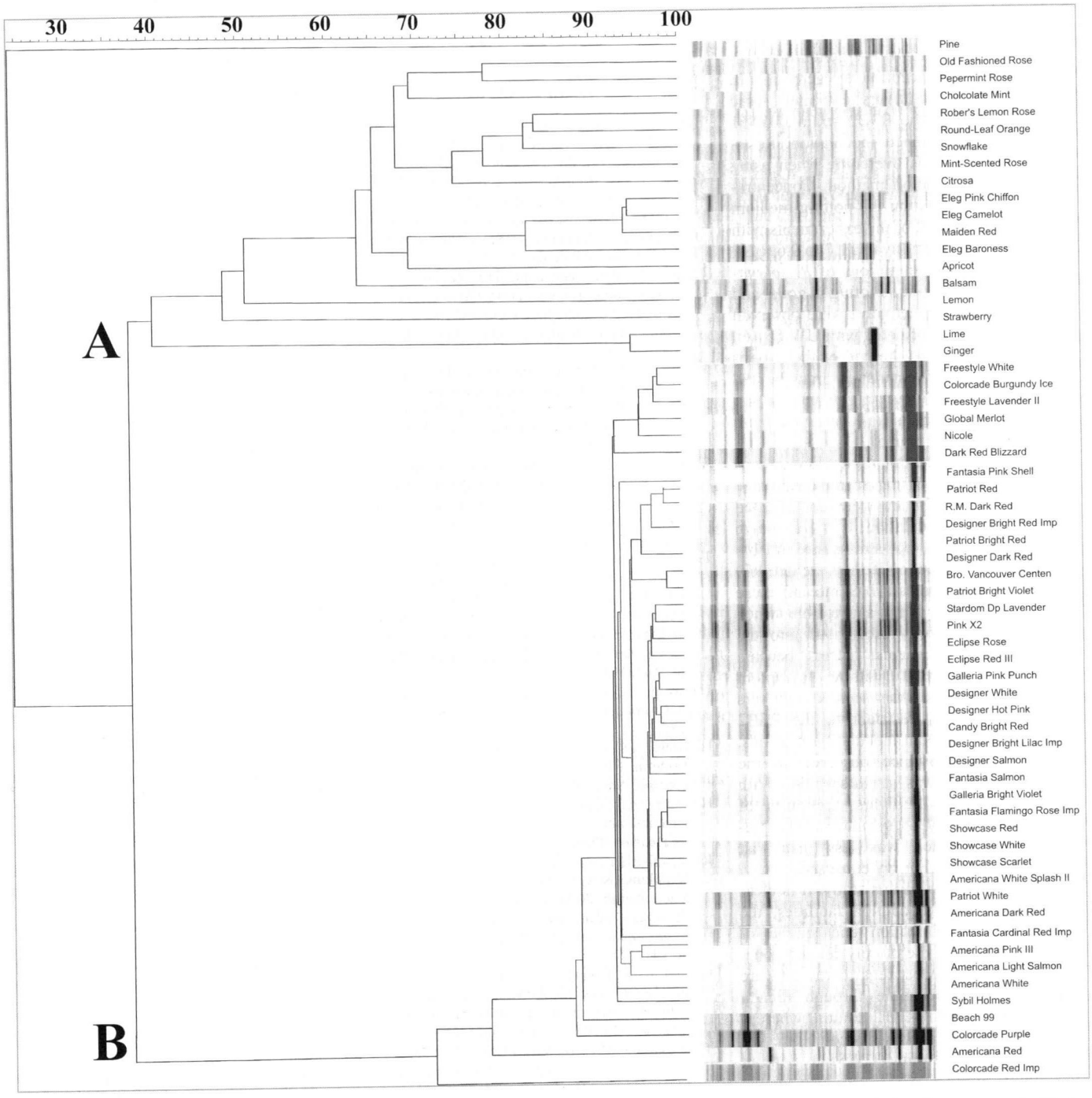

Fig. 1. Genetic comparison of 61 cultivars of geraniums using amplified fragment length polymorphism. Similarity coefficients displayed across top axis. Cluster A contains regal and scented cultivars. Cluster B contains all ivy and zonal geranium cultivars. Scented cultivar Pine was an outlier separate from both clusters.

Table 2. Populations of *R. solanacearum* in potting medium used for each geranium type.[z]

Strain	Zonal	Ivy	Regal	Scented
UW551	9.0×10^5 cfu/g a	9.6×10^5 cfu/g a	0 a	5.0×10^4 cfu/g a
P597	1.7×10^5 cfu/g a	1.7×10^5 cfu/g a	1.4×10^5 cfu/g b	3.3×10^3 cfu/g a
P673	2.8×10^5 cfu/g a	2.0×10^5 cfu/g a	2.2×10^4 cfu/g ab	2.7×10^5 cfu/g a

[z]Concentrations in columns of *R. solanacearum* associated with each geranium type followed with a different letter are significantly different ($P = 0.05$).

degree of resistance to infection with either R1B1 or R3B2 strains or both. Even under wound inoculation conditions, certain cultivars were highly resistant to infection with either the R1B1 (P673) or R3B2 (UW551) strain (Table 1). *R. solanacearum* could not be isolated from the soil or plant stem of certain scented cultivars. Some of the other scented cultivars only became infected through wound inoculation. Other scented geranium cultivars became systemically infected, yet no bacterial wilt symptoms were evident. No significant difference was observed in soil populations of the different strains of *Ralstonia* among scented cultivars (Table 2).

Discussion

Sixty-one cultivars representing four types of geraniums were tested for their susceptibility to three different strains of *R. solanacearum* to facilitate the management of bacterial wilt of geranium and to gain a

better understanding of the disease risk posed by the select agent UW551.

All 32 zonal and 10 ivy geranium cultivars tested were susceptible to bacterial wilt. A few of these cultivars exhibited some resistance toward the R3B2 strain UW551 applied through drench inoculation. Resistance, however, was overcome when using the wound inoculation method. Concentrations of bacteria within the potting medium were high at the end of the experiments with both zonal and ivy cultivars (1.7 to 9.6×10^5 cfu/g). Higher concentrations of *R. solanacearum* (9.0 and 9.6×10^5 cfu/g in zonal and ivy geraniums, respectively) were observed in potting media inoculated with UW 551 60 d after inoculation at the end of the experiment. This higher population may be the result of slower disease onset and, subsequently, slower plant tissue decay and release of bacteria into the media.

Resistance to natural root infection was observed in many of the scented cultivars. Many of these cultivars were not infected through drench inoculation. It is not known whether the *Ralstonia* strains are unable to colonize the roots or if they are actively killed by root exudates. Some of these resistant cultivars were susceptible when bacteria were directly inoculated into the vascular system, whereas others became systemically infected without symptom expression. Most of the scented cultivars tested were highly resistant to the R3B2 strain UW551 by both inoculation methods. This type of resistance observed in the scented cultivars was strain-specific. With regal and scented cultivars, the onset of symptoms, severity of symptoms, and frequency of infection was less than that observed with zonal or ivy cultivars.

From the results of this study, it is clear that in geranium production facilities, the greatest risk of infection and spread of bacterial wilt is in the closely related zonal and ivy cultivars. Most regal cultivars were shown to be susceptible through drench inoculation to both R1B1 strains, whereas most regal cultivars did not become infected with the R3B2 strain (UW551) without wounding. Strain UW551 was also unable to colonize root systems of the regal cultivars. Scented cultivars are also less likely to become infected through either root infection or cutting utensils. Bacterial populations in the media of scented cultivars were specific to strain and cultivars with large variations in population. However, if a bacterial wilt outbreak occurs within a production facility, all geranium cultivars should be suspected of infection as a result of the possibility of latent infections or asymptomatic plants.

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